A FRET based approach to study the SUMOylation of Serotonin 1A Receptors

By

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Submitted to the graduate degree program in Pharmacology and Toxicology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science.

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Date Defended: June 07, 2018

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Date Approved: June 7, 2018

ABSTRACT

Serotonin 1A receptors are an inhibitory G-protein coupled receptor that are known to play a key role in the regulation of mood and cognition. Dysregulation of serotonin 1A receptors has been implicated in mood related disorders such as depression and anxiety. Post translational modifications including palmitoylation and phosphorylation are found to regulate the function of serotonin 1A receptors. Previous studies in our lab demonstrated that serotonin 1A receptors are SUMOylated, however the impact of SUMOylation on serotonin 1A receptor function is yet to be elucidated. Acute agonist stimulation of serotonin 1A receptors was found to increase the levels of the SUMOylated receptors in rat cortex. This study employed acceptor photobleach FRET to further investigate the interaction between SUMO-1 and serotonin 1A receptor and identify the sites of SUMOylation on the serotonin 1A receptor. We used cell lines expressing both endogenous (N2A) and transfected serotonin 1A receptors (HEK293 and SHSY5Y) and observed FRET between serotonin 1A receptor and SUMO-1 in all the cell lines. Using acceptor photobleach FRET, we found three lysine residues on the serotonin 1A receptor (232, 235, 324) that are possibly involved in SUMOylation. We also conducted an immunocytochemistry-based approach, to study the effect of agonist stimulation of serotonin 1A receptors on SUMOylation of the receptors in the cell membrane. We observed similar extent of colocalization of serotonin 1A receptor and SUMO-1 antibody in both the 8-OH-DPAT and vehicle treated groups. This was observed due to the limitations of light microscopy to distinguish between objects closer than 100nm as two different entities. Further studies need to be performed using techniques with higher resolution such as electron microscopy to study the effect of agonist stimulation on the SUMOylated serotonin 1A receptors. Our data provides some important insights about the putative sites of SUMOylation on the serotonin 1A receptor. The identification of the primary

site of SUMOylation along with the knowledge about the effect of agonist stimulation on the SUMOylation of serotonin 1A receptors would help us decipher the role of SUMOylation in serotonin 1A receptor desensitization. Further understanding the regulation of serotonin 1A receptors by SUMOylation will aid in elucidating the role of serotonin 1A receptors in various mood disorders such as depression.

ACKNOWLEDGEMENTS

First, I would like to thank my advisor, Dr. Nancy Muma for always guiding and helping me through this project. Her constant support helped me persevere through all the problems we faced going forward with the project. Her creativity in scientific writing has been nothing but inspirational. I am grateful and blessed to have gotten a chance to work for her. I would like to extend my thanks to Dr. Eduardo Rosa-Molinar, for helping me discover this passion for microscopy that I did not even realize I had. He has been a constant support and has always pushed me on to do better things. I will always be grateful for all the encouraging words and the strong belief he had in me. I would also like to thank Dr. Honglian Shi for always being patient and supportive.

Next, I would like to thank my current and former lab members. They have been my constant support system. Khushboo Kapadia has been my companion throughout, from all the hard and trying times to times when we would just sit and laugh all day. Sugandha has always helped cheer me up in tough times. Amanda and Yusheng, had been helpful and always brought a smile on my face. I would also like to extend my thanks to my FRET buddy, Heather, who has helped me sift through this project rather smoothly.

Next, I would like to thank my family and friends. My friends for helping me find a home away from home and my family for making me feel that home is not that far away.

Last but not the least, I would like to thank the funding source. This project was funded by JR+Inez Jay Fund, Higuchi Bioscience Centre and Strategic Initiative Grant from the Provost's office, KU.

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1. INTRODUCTION

1.1 SEROTONIN

Serotonin also known as 5-hydroxytryptamine (5-HT) is a monoamine, which acts as a neurotransmitter in the central nervous system(CNS). The initial step in synthesis of serotonin in the CNS is the transport of tryptophan from blood to brain. Serotonergic neurons express the enzyme tryptophan hydroxylase-2 which catalyzes the hydroxylation of tryptophan to 5-hydroxytryptophan.This is the rate limiting step in the synthesis of 5-HT. Aromatic amino acid decarboxylase then catalyzes the decarboxylation of 5-hydroxytryptophan to 5-hydroxytryptamine (5-HT)[1]. Metabolism of 5-HT involves its deamination to 5-hydroxyindoleacetaldehyde which is catalyzed by monoamine oxidase. 5-hydroxyindoleacetaldehyde can further be oxidized to 5-hydroxyindoleacetic acid (5-HIAA)[2].

5-HT is involved in the regulation of various processes in the brain, such as mood, emotions, aggression, sleep, appetite, memory, and perceptions[3]. 5-HT regulates these processes through different pathways that innervate various brain regions. Most of the cells in the brain, are either directly or indirectly affected by 5-HT levels. Abnormal 5-HT levels are associated with mood and anxiety disorders, depression, panic attacks, and insomnia[4].

5-HT produces a myriad of physiological effects which are mediated through fourteen distinct receptor subtypes, out of which thirteen are G-protein coupled receptors and one is a ligand gated ion channel. These receptors can be broadly classified into four subfamilies: the 5-HT1 family, the 5-HT2 family, the 5-HT4, 5-HT5, 5-HT7 family, all of which are coupled to G-proteins. The fourth family has only one receptor subtype, 5-HT3 receptor which is a homomeric ligand gated ion channel[5]. 5-HT1 family consists of 5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E and 5-HT1F receptors. These receptors are predominantly coupled to the Gi/Go pathway and their

activation results in the inhibition of adenylyl cyclase. 5HT1A receptors are also known to couple to Gαz proteins in the hypothalamus and mediate the release of hormones[6]. The 5-HT2 family consists of 5-HT2A, 5-HT2B and 5-HT2C receptors. These receptors are coupled to Gq/11 pathway and their activation results in the stimulation of phosphoinositide specific phospholipase C (PI-PLC). The third family consists of 5-HT4, 5-HT5 and 5-HT7 receptors; these receptors couple to Gαs proteins and their activation causes the stimulation of adenylyl cyclase. The 5-HT3 receptor is a serotonin-gated cation channel that causes rapid depolarization of neurons.

1.1.1 SEROTONIN 1A RECEPTORS (5HT1AR)

The 5HT1AR is a G-protein coupled receptor subtype that exists in two major forms in the nervous system: the pre-synaptic auto-receptor and the post-synaptic receptor. 5HT1A auto-receptors reside on the soma and dendrites of serotonergic neurons in the raphe nuclei, where its activation results in the hyperpolarization and reduction in the firing rate of these neurons, and thereby reducing the extracellular levels of serotonin.

Postsynaptic 5HT1AR are expressed in the areas receiving serotonergic innervation. These receptors are mainly located on pyramidal neurons and on GABAergic neurons. They are highly expressed in brain regions implicated in the regulation of mood and anxiety, such as the frontal cortex, hippocampus , hypothalamus and amygdala[7]. The postsynaptic 5HT1AR is coupled to the family of Gi/Go proteins and their downstream effector systems. Two of the many downstream signaling pathways include: (i) inhibition of adenylyl cyclase activity and (ii) the opening of K^+ channels, which results in neuronal hyperpolarization. In terminal field areas of serotonergic innervation, such as the hippocampus, 5HT1ARs are coupled to both effector

systems. However, in the dorsal raphe nucleus, 5HT1ARs are coupled only to the opening of potassium channels[8].

Dysregulation of 5HT1ARs occurs in patients suffering from depression and related mood disorders[9, 10]. Increases in 5HT1A auto-receptor density in the midbrain have been demonstrated in depressed suicide patients[10, 11]. 5HT1ARs have been also examined in many cerebral cortical and subcortical areas in patients with a history of mood disorders[12].Positron emission tomography (PET) studies revealed decreased 5HT1AR levels in the cortex in untreated or treated depressed patients. Decreased 5HT1AR levels were also observed in patients with remitted depressive episodes[13]. Furthermore, reduced 5HT1AR levels have also been reported in patients with social anxiety disorders and in cortical regions of patients suffering from panic disorder, although not all studies agree[14]. However, the overall evidence suggests that 5HT1AR function is altered in clinical populations when compared to controls. Furthermore, it should be noted that the observed abnormalities in 5HT1AR levels are found in many affective and anxiety-related disorders, suggesting that these findings may reflect a general vulnerability factor for psychopathology.

In addition to the human studies, numerous studies in transgenic and knockout animals have also been performed to study the function of 5HT1AR[15-17]. 5HT1AR knockout mice exhibited an anxiety-like phenotype in behavioral tests such as open field, elevated plus maze, and novelty-suppressed feeding test[17]. The impaired performance of 5HT1AR knockout mice was observed to be due to an enhanced fear response, but not due to a deficit in exploratory drive[18]. Interestingly, despite the association of 5HT1AR function with depression in humans, 5-HT1AR knockout mice did not display a prominent depression-like phenotype. Moreover,

5HT1AR KO mice display increased physiological responses to acute stress. However, these behavioral alterations are not correlated with 5-HT or 5-HIAA levels in brain tissue[19, 20].

In contrast to the behavioral changes observed in mice lacking the 5HT1AR, a transgenic mouse model overexpressing 5HT1AR in the central nervous system under control of its endogenous promoter had reduced anxiety-like behavior, reduced 5-HIAA/5-HT ratio in several brain areas and elevated serotonin levels in the hippocampus and striatum[21]. The data from this study suggests that overexpression of 5HT1ARs results in an opposite phenotype of 5HT1AR knockout mice.

Another transgenic mouse model that was used to independently assess the function of 5HT1AR auto-receptors and post-synaptic receptors provided a number of advances over classic KO and previous transgenic approach. The study demonstrated that suppression of post-synaptic 5HT1A receptors is not sufficient to impact anxiety-like behavior. However, loss of auto-receptors impacts anxiety, suggesting that the anxiety-like phenotype of the 5HT1AR KO mouse likely results from increased serotonergic neuron excitability due to the loss of auto-receptors. Furthermore, mice lacking post-synaptic 5HT1ARs displayed decreased mobility in the forced swim test indicating an increase in behavioral despair which is consistent with depressive-like phenotype. In contrast, loss of 5HT1A auto-receptors did not impact behavior in the forced swim test[22]. These results provide evidence for distinct roles of the 5HT1A auto-receptors and post-synaptic receptors in mediating anxiety and depressive like phenotypes in mouse models, suggesting that auto receptors impact the anxiety-like behavior and the post-synaptic receptor impacts the depressive-like behavior.

1.1.2 DEPRESSION

Depression is a mood disorder that is characterized by a sense of insufficiency, despair, decreased activity and anhedonia, to an extent that these symptoms adversely affect a person's life. The World Health Organization (WHO) estimates depression to be the leading cause of disability worldwide and a major contributor to the overall global burden of diseases. Currently about 16.1 million people in the United States are suffering from major depressive disorder. The prevalence is higher in females as compared to males, one in every five women and every twelve men are diagnosed with depression.[23]

As described in the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM- V), the hallmark of major depressive disorder is the occurrence of depressed mood and loss of interest in activities that were rather pleasurable in the past (anhedonia), with the symptoms lasting for at least a duration of two weeks. These symptoms must also be accompanied by at least four of the following indicators: changes in appetite or weight, changes in sleep patterns, altered psychomotor activity, feelings of worthlessness, difficulty concentrating or making decisions and recurrent thoughts of death[24].

There are many forms of depression ranging from mild to severe, two of the most common forms are major depressive disorder and dysthymic disorder. In major depressive disorder, patients typically show symptoms of depressed mood and anhedonia along with alterations in sleep patterns, appetite, and disturbances in cognitive functions. These symptoms severely affect the patient's daily life. In dysthymic disorder, also known as the pervasive depressive disorder, the patient exhibits depressive mood for majority of the day for at least two years. This is in contrast to major depression in which the patient has a discrete episode of severe depression, after which the patient is in "remission" and feels normal. Some other forms of

depression are slightly different and may develop only under unique situations such as seasonal affective disorder, where the patient only gets depressive episodes in a particular season such as winters and post-partum depressive disorder which affects mothers after giving birth[25].

Even though many studies have tried to shed light on the pathophysiology of depression, it still is elusive. There are however many theories that explain the pathogenesis based on indirect markers and post mortem reports of depressive patients. One of the major theories of the pathophysiology of depression is the "Serotonin Hypothesis". The serotonin hypothesis postulates that there is a net reduction in the levels of serotonin in depressive disorder[26]. This reduction in serotonin levels can be either due to decreased serotonin availability or it can be due a defect in the receptor activity. The basis for this hypothesis is the fact that the first antidepressant drugs worked by increasing serotonin activity in the brain[27]. Studies have shown that low dietary tryptophan has an anxiogenic and depressant effect on rat behaviors, where the rats display immobility in the forced swim test and anxiety related behaviors in the elevated plus maze[28]. The decrease in plasma level of tryptophan has also been shown to induce depressive symptoms in elderly patients[29]. A subset of depressed patients has been reported to have lower levels of 5-hydroxyindoleacetic acid (5-HIAA) a metabolite of 5-HT in the cerebrospinal fluid (CSF), which has been related to aggressive behavior and increased suicidal tendency[30]. Moreover, positron emission tomography (PET) imaging studies have reported a decrease in density of 5HT1AR in depressed patients in different regions of the brain.[31, 32]

1.1.3 ANTIDEPRESSANTS

Antidepressants are drugs used for the treatment of major depressive disorder and other conditions, including dysthymia, anxiety disorders, obsessive–compulsive disorder, attention-

deficit hyperactivity disorder (ADHD), and sleep disorders. They may be prescribed alone or in combination with other medications. Antidepressant drugs either inhibit the reuptake or decrease the metabolism of monoamines in the CNS[33]. There are several classes of antidepressant drugs. Tricyclic antidepressants (TCA) inhibit the reuptake of neurotransmitters such as serotonin and norepinephrine. TCAs were the first drugs for depression and although they are effective, they have serious side effects and can be lethal in overdoses. Some of the classic examples of drugs from this class are amitriptyline, desipramine and imipramine[34]. Monoamine oxidase(MAO) inhibitors prevent the metabolism of monoamines by monoamine oxidase. As a result, the levels of neurotransmitters such as serotonin, dopamine and norepinephrine increase. The downside is that MAO inhibitors also prevent the body's ability to break down other medicines metabolized by this enzyme as well as the amino acid tyrosine, which is found in foods such as cheese. Some typical examples of MAO inhibitors are selegline and isocarboxazid[35].

Reuptake inhibitors reduce the transport of neurotransmitters from the synapse into the presynaptic neurons. There are three types of reuptake inhibitors, serotonin and norepinephrine reuptake inhibitors (SNRI), norepinephrine and dopamine reuptake inhibitors (NDRI) and selective serotonin reuptake inhibitors (SSRI). SNRIs prevent the reuptake of serotonin and norepinephrine by blocking the serotonin transporter (SERT) and norepinephrine transporter (NET). Some examples include venflaxine and duloxetine. NDRIs inhibit the reuptake of norepinephrine and dopamine by blocking the NET and dopamine transporter (DAT). There is only one drug in this class, bupropion. SSRIs are the most common antidepressants that are prescribed. SSRIs prevent the reuptake of serotonin by blocking the serotonin transporter (SERT). Fluoxetine, paroxetine and citalopram are some examples of drugs of this class[36].

The mechanism by which the antidepressants increase the levels of neurotransmitters is known but how they alleviate the depressive symptoms is yet to be elucidated. For example, SSRIs rapidly block the SERT to increase the 5-HT levels, however it takes about 6-8 weeks for the antidepressant effects of the drugs to appear[37]. This lag might be due to receptor desensitization or some other adaptive changes. 5HT1A auto-receptors are thought to limit the initial increase of 5-HT levels induced by SSRIs, thus delaying the therapeutic response. The effect is gradually overcome by desensitization of 5HT1A auto-receptors in the raphe nuclei, allowing the firing rate of serotonergic neurons to recover. SSRIs have been shown to increase 5-HT levels in both the frontal cortex and raphe nuclei areas, and this effect is greater in mice with global 5HT1AR KO mice[38]. Interestingly, mice with conditional 5HT1AR KO in the hippocampus respond to tricyclic antidepressants (TCAs), but not to the SSRI fluoxetine, in the tail suspension test and the novelty suppressed feeding test, suggesting that the 5HT1A receptors are a critical component in the mechanism of action of SSRIs in the hippocampus but not TCAs[39].

1.2 POST TRANSLATIONAL MODIFICATIONS OF 5HT1ARs

5HT1ARs are known to undergo a number of post-translational modifications (PTMs), namely palmitoylation, phosphorylation and SUMOylation. Modification of 5HT1ARs by palmitic acid was studied using pulse-chase experiments, where it was observed that fatty acids were stably attached to the cysteine residues 417 and 420 of the receptor and studies also suggest that it is probably an irreversible modification[40]. Palmitoylation of 5HT1AR could affect the receptor function in one of the two ways: it might be required for the interaction of the receptor to the G-proteins or that it could be involved in the trafficking of the receptor to the membrane subdomains such as lipid rafts or detergent resistant microdomain[41]. Phosphorylation of

5HT1ARs mediated by protein kinase C has been shown to cause the desensitization of the receptor[42]. Our lab has recently discovered that 5HT1ARs also are SUMOylated.

1.2.1 SUMOylation

SUMOylation is a post-translational modification that involves the covalent attachment of a member of the small ubiquitin-like modifier(SUMO) family of proteins to lysine residues in specific target proteins via an enzymatic cascade similar to the ubiquitination pathway. In mammals there are four SUMO paralogues, SUMO-1 to SUMO-4. SUMO-1 is an 11 kD protein that was isolated as the binding partner of RAD51/52 nucleoprotein filament proteins, which mediate DNA strand exchange[43]. The first reports of SUMO-1 functioning as a covalent protein modifier described SUMOylation of the nuclear pore protein Ran-GTPase-activating protein 1(RANGAP-1)[44]. SUMO-2 and SUMO-3 differ from each other by only three N-terminal residues and are often referred to collectively as SUMO-2/3. SUMO-2/3, however, share only 50 % similarity with SUMO-1. Interestingly, SUMO-2/3 can form chains on substrate proteins through internal lysine residues, while SUMO-1 only forms monomers (monoSUMOylation) on the substrate and appears to act as a chain terminator on SUMO-2/3 polymers. In contrast with the other SUMO genes the SUMO-4 gene lacks introns, raising the possibility that it may be a pseudogene. However, recent studies show that SUMO-4 is expressed in normal placental development. SUMO-4 expression was increased in pre-eclamptic placentas and in models of oxidative stress and hypoxic injury suggesting that SUMO-4 hyper-SUMOylation may be a potential post-translational mechanism in the stressed pre-eclamptic placenta[45].

Protein modification by SUMO may lead to one of the following effects on the substrate proteins; first, SUMOylation may mask the binding site of a protein that interacts with the substrate protein, essentially occluding the interaction in a SUMO-dependent manner. For

example, SUMOylation of the ubiquitin-conjugating enzyme E2-25K inhibits its interaction with ubiquitin E1 enzyme, leading to a decrease in the ubiquitin conjugation of substrates[46]. Second, the covalently attached SUMO may act as a hub that recruits new interacting proteins to the substrate either by a direct interaction with the SUMO moiety or by an indirect interaction at the SUMO-substrate interface. For example, SUMOylation of RANGAP-1 promotes its interaction with Ran binding protein 1 and its relocation from the cytoplasm to the nuclear pore complex[44]. Third, SUMOylation can lead to a conformational change in the SUMOylated substrate, directly regulating its function. [47].

1.2.2 THE SUMOylation CYCLE

Conjugation

SUMOylation is mediated through an enzyme cascade similar to ubiquitination as it involves the activation, conjugation and ligation of SUMO protein to its substrate. SUMO proteins are synthesized as inactive precursors that must first undergo a C-terminal cleavage catalyzed by a sentrin/SUMO-specific protease(SENP) enzyme. This cleavage exposes a di-glycine residue that allows SUMO to be conjugated to lysine residues in target proteins. During each conjugation cycle, SUMO proteins are first activated in an ATP-dependent manner by the E1 ‘activating’ enzyme which is a heterodimer of SUMO-activating enzymes (SAE1 and SAE2) in mammals. This step involves the formation of a thioester bond between the active-site cysteine residue of SAE2 and the C-terminal glycine residue of SUMO[48].

SUMO is then passed to the active cysteine residue of the conjugating enzyme ubiquitin-conjugating 9 (Ubc9), again via a thioester linkage. Importantly, Ubc9 is the only known SUMO-conjugating enzyme and Ubc9 can also bind directly to the SUMOylation consensus motif on substrate proteins. Transfer of SUMO from Ubc9 to the target protein can

occur either independently through the direct interaction of Ubc9 with the substrate or in the presence of a ligase enzyme which facilitates SUMOylation of the substrate[47]. SUMO transfer from Ubc9 to a target protein can occur through two ligase-independent mechanisms. First, many SUMOylated lysine residues lie within a consensus motif, Ψ-K-x-[D/E] (where Ψ is an aliphatic branched amino acid, K is the lysine residue where the SUMO protein binds, x is any amino acid and D/E represent an acidic amino acid such as aspartate and glutamate). Ubc9 can directly recognize this motif and conjugate the lysine residue within it. Alternatively, some SUMO substrates have SUMO interaction motifs (SIMs) that promote their own conjugation. These SIMs bind to the SUMO moiety to which Ubc9 is attached, thereby increasing the local concentration of SUMO and facilitating SUMOylation[49].

Ligase-dependent transfer of SUMO from Ubc9 occurs in the presence of E3 enzymes which facilitate majority of SUMOylation reactions under physiological conditions. One such family of E3 ligases is that of protein inhibitor of activated STAT (signal transducer and activator of transcription) [PIAS] proteins. The PIAS family consists of five members, each of which has ligase activity. The PIAS proteins have a SP (Siz/PIAS)-RING domain similar to the ones found in many ubiquitin E3 ligases. SP-RING ligases bind to Ubc9, their substrate proteins and bind non-covalently to SUMO, thus acting as a scaffold bringing SUMO-loaded Ubc9 together with substrate proteins. In addition to the PIAS proteins, a number of other SP-RING domain-containing proteins have been reported to function as SUMO E3 ligases including Ran-binding protein 2[50].

DeSUMOylation

Protein SUMOylation is a highly dynamic process that can be readily reversed by the action of the SENP enzyme, which was initially needed for the maturation of SUMO precursor.

Mammals have six SENPs, designated as SENP1–3 and SENP5–7. These SENPs vary in their cellular distribution, SUMO paralogue specificity and selectivity for SUMO maturation compared with deconjugation activities. Mammalian SENPs can be classified into three groups. SENP1 and SENP2 have a broad specificity for SUMO-1 and SUMO-2/3 and function both in their maturation and deconjugation. SENP3 and SENP5 favor SUMO-2/3 over SUMO-1, i.e. they function in the removal of monomeric SUMO-2/3 chains. SENP6 and SENP7 also act preferentially on SUMO-2/3. Neither SENP6 nor SENP7 seem to be involved in maturation of pro-SUMO proteins and they show minimal activity in the deconjugation of monomeric SUMO-2/3 from substrate proteins. Rather, SENP6 and SENP7 efficiently edit and deconjugate poly-SUMO-2/3 chains[51].

1.2.3 FUNCTIONS OF SUMOylation

Depending on the target protein, SUMOylation can occur at the cell membrane, cytoplasm or nucleus, and is involved in regulating the subcellular localization of a number of substrate proteins.

Nuclear Functions:

SUMOylation is known to be a predominantly nuclear modification and is known to modify a large number of chromatin-remodeling complexes. RANGAP-1 was the first identified SUMO substrate and plays an important role in the regulation of transport of ribonucleoproteins and proteins across the nuclear pore complex. Unmodified RanGAP1 resides predominantly in the cytoplasm and upon conjugation with SUMO associates with the cytoplasmic fibers of the nuclear pore complex[44, 52]. The promyelocytic leukemia and Sp100 proteins are major components of promyelocytic leukemia nuclear bodies, also called nuclear domain 10.

SUMOylation has been found to be required for the subcellular localization of most of the proteins found in nuclear domain 10 such as promyelocytic leukemia[53].

The SUMOylation of transcription factors has been reported to have different effects on their activities in various pathways including those involving cytokines, WNT, steroid hormones, and AP-1. In most cases, SUMO modification plays a negative role in transcription regulation[54, 55]. Although SUMOylation of most transcription factors results in repression, it appears to have positive effects on transcriptional activation by the heat shock factors HSF1 and HSF2. SUMOylation of HSF1 and HSF2 is correlated with their localization to promyelocytic leukemia nuclear bodies[56, 57]. For both HSFs, SUMOylation leads to increased DNA-binding activity.

SUMOylation also plays an important role in cell cycle and division such as chromosome cohesion and kinetochore assembly. During mitosis, the proper distribution of chromosomes into replicated cells is dependent upon the sister chromatid assembly and separation. Dysregulation of this process was one of the first phenotypes described in SUMO-1 mutants in yeast and is characterized by aberrant mitosis and defects in chromosomal segregation[58].

Extranuclear Functions:

The nuclear functions of SUMOylation have been very well characterized, but recent studies indicate many important roles for SUMOylation in signal transduction, trafficking and modification of cytosolic and membrane proteins. Phosphorylation cascades are integral to cell signaling, and important elements of these pathways can be modulated by SUMOylation. For example, focal adhesion kinase and protein tyrosine phosphatase 1b are both SUMO substrates. SUMOylation of focal adhesion kinase results in its autophosphorylation and that of protein tyrosine phosphatase 1b results in transient downregulation of both its activity and expression[59,

[60]. Mitochondrial fission is dependent on the GTPase dynamin-related protein 1 and SUMOylation of dynamin-related protein 1 appears to protect it against degradation[61].

In addition to proteins involved in cell signaling, many membrane-associated proteins have also been observed to be SUMOylated. Glucose transporters GLUT1 and GLUT4 were the first membrane proteins that were found to be SUMOylated[62]. However, the functional effect of SUMOylation on these transporters is still unclear. SUMO conjugation has also been shown to regulate the function of two neuronal K^+ channels, K2P1 and Kv1.5, indicating the role of SUMOylation in neuronal excitability[63]. Kainate receptors are tetrameric glutamate-gated ion channels that undergo activity-dependent SUMOylation. Kainate receptors are selectively and rapidly SUMOylated in response to agonist activation, leading to the endocytosis of the receptor[64].

In addition to the above-mentioned membrane associated proteins, recently many proteins associated with G-protein signaling have also been found to be SUMOylated. Metabotropic glutamate receptors(mGLuRs) are one of the first G-protein coupled receptors that were shown to be SUMOylated. Although the exact function of SUMOylation of mGLuRs is still unclear, it is thought that SUMO might provide an interface for the binding of many interacting proteins[65]. Another G-protein coupled receptor that is known to be SUMOylated is cannabinoid receptor 1(CB1). It was found that SUMOylation of CB1 is decreased upon agonist treatment[66]. The regulators of G-protein signaling, RGSZ1 and RGSZ2 are SUMOylated upon the activation of μ-opioid receptors. SUMOylation of RGSZ1 and RGSZ2 is thought to mediate the desensitization of G-protein coupled receptors[67]. Another G-protein coupled receptor that has been found to get SUMOylated is 5HT1A receptor[68].

1.2.4 SUMOylation OF 5HT1ARs

Previous studies from our lab reported that 5HT1ARs are SUMOylated by SUMO-1 proteins[68]. Using immunoprecipitation followed by immunoblotting for, SUMOylated 5-HT1ARs were detected in the membrane fraction but not in the cytosolic fraction of the rat cortex. These SUMOylated 5HT1ARs were found in numerous brain regions such as frontal cortex, cortex, hippocampus, hypothalamus, midbrain, and the dorsal raphe. Most of the brain regions had comparable expression of SUMOylated 5HT1ARs, except for the midbrain which had a lower expression. Results also showed that acute treatment with 8-hydroxy-2-dipropylamino tetralin (8-OH-DPAT), an agonist of the 5HT1AR, significantly increased the expression levels of SUMOylated 5HT1ARs.

Active 5HT1ARs are located in the detergent resistant membrane microdomain (DRM). To determine if the SUMOylated 5HT1ARs are located in the DRM, our lab previously isolated the DRM by cold triton-X100 treatment followed by sucrose gradient centrifugation. The majority of SUMOylated 5HT1AR was found in the triton-X100 soluble fraction and very low levels of SUMOylated 5HT1ARs were observed in the DRM fraction. Moreover, acute treatment with 8-OH-DPAT increased the expression of SUMOylated 5HT1ARs in the DRM.

To further determine the subcellular location of the SUMOylated 5HT1AR, a discontinuous gradient centrifugation with 7.5-30% iodixanol was performed. The subcellular organelles located in the fractions of rat cortex were collected and confirmed by immunoblotting with several organelle markers. The SUMOylated 5HT1ARs were primarily found to be colocalized with TGN38, a marker for the transgolgi-network (TGN) and calreticulin, a marker for the endoplasmic reticulum (ER).

An 8-OH-DPAT binding assay was also conducted in the subcellular fractions to determine if the SUMOylated 5HT1ARs were functional. Subcellular fractions were obtained by discontinuous gradient centrifugation with 10-40% iodixanol and each fraction was divided into two. One half was used for the binding assay and the other half was used for immunoprecipitation of SUMOylated 5HT1ARs. It was observed that the fractions that had high 8-OH-DPAT binding had low levels of the SUMOylated receptors and the fractions with high levels of SUMOylated receptor showed low 8-OH-DPAT binding.

1.3 STATEMENT OF PURPOSE

From the previous studies in our lab, we know that 5HT1ARs are SUMOylated and acute treatment with a selective 5HT1AR agonist i.e. 8-OH-DPAT results in an increase in the levels of SUMOylated 5HT1ARs. Based on the previous studies suggesting various roles of SUMOylation in the regulation of the trafficking of 5HT1ARs and possibly in the 5HT1AR signal transduction, it is necessary to understand how SUMO-1 interacts with 5HT1AR. Thus, the first goal for this study is to identify the primary site of SUMOylation of 5HT1AR**.** Identification of the primary site of SUMOylation would help us understand the effect that SUMOylation may have on 5HT1AR function. Moreover, mutation of this primary site of SUMOylation would provide a tool to further investigate the role of SUMOylation in the 5HT1ARs signal transduction.

Our lab has previously studied the role of agonist stimulation in the SUMOylation of 5HT1ARs using molecular biology techniques and had observed an increase in the SUMOylation of 5HT1ARs in the membrane fractions after agonist stimulation. To verify the effects of agonist treatment on the SUMOylation of 5-HT1ARs, my second goal is to study the effect of acute agonist stimulation on the SUMOylation of 5HT1ARs using immunocytochemistry**.**

Overall, both the goals would help us to elucidate the role of SUMOylation in 5HT1AR function and relation between the SUMOylation of 5HT1ARs and receptor desensitization after stimulation by 5HT1AR agonists.

2. MATERIAL AND METHODS

2.1 Cell Culture

Mouse neuroblastoma cells (N2A) and human neuroblastoma cells (SHSY5Y) were maintained in 50% Dulbecco's minimum essential medium (DMEM high glucose, Catalog no. #11-995-073, Fisher Scientific, Waltham, MA, USA), 50% Opti MEM reduced serum media (Catalog no. #31-985-088, Fisher Scientific, Waltham, MA, USA), 10% fetal bovine serum (FBS) (Catalog no. #S11150, Atlanta Biologicals, Flowery Branch, GA, USA) and 1% penicillin-streptomycin (Catalog no. #P0781-100ML, Sigma Aldrich, St. Louis, MO, USA). Human embryonic kidney cells (HEK293) were maintained in Eagle's minimum essential media (EMEM with L-Glutamine, Catalog no. #30-2003, ATCC, Manassas, VA, USA) and 10% FBS (Catalog no. #S11150, Atlanta Biologicals, Flowery Branch, GA, USA). For immunocytochemistry, N2A cells were plated in either micro-slide 8-well glass bottom plates (Catalog no. #80827, Ibidi, Martinsried, Germany) at a seeding density of 20,000 cells per well; or in 96-well glass bottom plates (Catalog no. #P96-1-N, CellVis, Mountain View, CA, USA) at a seeding density of 15,000 cells per well. The plates were coated with 10% poly-L-lysine (Catalog no. #P8920-100ML, Sigma Aldrich, St. Louis, MO, USA). SHSY5Y cells were plated in micro-slide 96-well plates (Catalog no. #89626, Ibidi, Martinsried, Germany) coated with 10µg/ml fibronectin (fibronectin bovine plasma, Catalog no. #F1141-1MG, Sigma Aldrich, St. Louis, MO, USA) at a seeding density of 20,000 cells per well. HEK293 cells were plated in micro-slide 8-well polymer coverslip plates (Catalog no. #80826, Ibidi, Martinsried, Germany) at a seeding density of 15,000 cells per well. N2A cells were treated with vehicle or 0.2 mg/ml 8-OH-DPAT for 5 mins, 15 mins, 30 mins, or 60 mins and were then fixed with 2% paraformaldehyde for 20 mins for immunocytochemistry.

2.2 Plasmid Constructs

To identify the primary SUMOylation site on 5HT1ARs we used plasmids constructed to express 5HT1ARs which have mutations on the putative sites of SUMOylation. Most SUMO substrates have a common SUMO recognition motif, Ψ-K-x-D/E, where Ψ is a hydrophobic residue consisting of 3–4 aliphatic residues, K is lysine conjugated to SUMO, x could be any amino acid (aa), D/E is an acidic residue. Based on the SUMO recognition motif above our lab had previously identified 5 putative SUMOylation sites on 5HT1AR. Using site specific mutagenesis mutant plasmids were constructed to express 5HT1ARs in which the lysine residues in the putative SUMOylation sites were mutated to arginine. The plasmid constructs used the pcDNA4hismaxc (Addgene, Cambridge, MA, USA) vector backbone and expressed either the wild type 5HT1AR (rat), or the mutant 5HT1ARs. The mutations are described as "KXR", where K is lysine, R is arginine and X indicates the position in the amino acid sequence of 5HT1AR where lysine was mutated to arginine. The plasmid constructs used expressed either the wild type 5HT1ARs or one of the following mutations in the 5HT1AR; K302R, K332R, K302.332R, K324R, K232.235R and K232.235.324R.

2.3 Transfection

SHSY5Y were transfected 16-24 hours after plating with 150ng of plasmids expressing either the wild type or mutant 5HT1ARs using lipofectamine 2000 (Catalog no. #11668019, ThermoFisher Scientific, Waltham, MA, USA). The media was changed once 3-4 hours after transfection. HEK293 cells were co-transfected with 125 ng wild type 5HT1ARs and 125 ng of Ubc9 (HEK293 cells have low levels of Ubc9) 48 hours after plating using lipofectamine PLUS reagent (Catalog no. #11514015, ThermoFisher Scientific, Waltham, MA, USA). The media was

changed once after 4 hours of transfection. 48 hours after the transfection, both the cells lines were fixed in 2% paraformaldehyde for 20 mins, and then used for immunocytochemistry.

2.3 Immunocytochemistry for Light Microscopy

SHSY5Y, N2A and HEK293 cells were rinsed twice with Hank's balanced salt solution (1X HBSS without calcium, magnesium, and phenol red, Catalog no. #21-022-CV, Nalgene, ThermoFisher Scientific, Waltham, MA, USA) and fixed in 2% paraformaldehyde in HBSS (PFA, Catalog no. #158127-500G, Sigma Aldrich, St. Louis, MO, USA) for 20 mins. After fixation, the cells were permeabilized with 1mg/ml saponin (Catalog no. #84510-100G Sigma Aldrich, St. Louis, MO, USA) in HBSS (HBSS-S) by rinsing three times for 5 minutes each. After permeabilization, the non-specific labelling of the cells was blocked in blocking buffer containing 3% normal goat serum (NGS, Catalog no. #31873, Invitrogen, ThermoFisher Scientific, Waltham, MA, USA), 0.02% triton-X100 (Catalog no. #T8787-100ML, Sigma Aldrich, St. Louis, MO, USA) in HBSS-S for 1 hour. The cells were then incubated with primary antibodies for the 5HT1AR and SUMO-1 diluted in the blocking buffer overnight at 4ºC. The next day, the cells were washed three times with HBSS-S for five minutes each and then probed with secondary antibodies conjugated to fluorophores diluted in blocking buffer and incubated for 2 hours at room temperature. After the incubation with secondary antibodies, cells were washed twice with HBSS-S for five minutes each and once with HBSS for five minutes. For SHSY5Y and HEK293, after the washes, prolong gold antifade reagent with DAPI (Catalog no. #P36931, ThermoFisher Scientific, Waltham, MA, USA) was added and at least 20 mins later, the cells were imaged. N2A cells, after the washes, were incubated with wheat germ agglutinin conjugated with Alexa fluor 488 (WGA) (Catalog No. #W1121, ThermoFisher Scientific, Waltham, MA, USA) for 30 minutes. The cells were rinsed with HBSS three times for five

minutes each. After the washes, prolong gold antifade reagent with DAPI was added and the cells were imaged.

2.4 Antibodies

The antibodies used in the experiments are listed below in Table 1.

Table 1: Summary of antibodies used

No.	Antibody	Antigen Sequence	Dilution	Catalog No.	Lot No.	Vendor
1.	Rabbit Polyclonal 5HT1AR	400-422 aa	1:200	PA1647	0161212c014731	Boster Bio, Pleasanton, CA, USA
2.	Rabbit Polyclonal 5HT1AR	294-312 aa	1:50	24504	1443001L	ImmunoStar, Hudson, WI, USA
3.	Mouse Monoclonal SUMO-1	1-101 aa	1:200	SC-5308	D0517	Santa Cruz, Dallas, TX, USA
4.	Mouse Monoclonal SUMO-1	85-97 aa	1:200	MA3-088	SH257204	Invitrogen, ThermoFisher Scientific, Waltham, MA, USA
5.	Goat anti rabbit IgG conjugated with Alexa Fluor® 568	-	1:200	A11036	1832035	Invitrogen, ThermoFisher Scientific, Waltham, MA, USA
6.	Goat anti mouse IgG conjugated with Alexa Fluor® 647	-	1:200	A21236	1654338	Invitrogen, ThermoFisher Scientific, Waltham, MA, USA
7.	Goat anti rabbit Fab' fragment conjugated with Alexa Fluor® 594	-	1:200	111-587-003	131732	Jackson ImmunoResearch, West Grove, PA, USA
8.	Goat anti mouse Fab' fragment conjugated with Alexa Fluor® 647	-	1:200	115-607-003	130849	Jackson ImmunoResearch, West Grove, PA, USA
9.	Alexa Fluor® 594 FluoroNanogold™ Fab' fragment of goat anti rabbit IgG	-	1:80	7304	33C047	Nanoprobes,Inc., Yaphank, NY, USA
10.	Alexa Fluor® 647 FluoroNanogold™ Fab' fragment of goat anti mouse IgG	-	1:80	7502	33C167	Nanoprobes,Inc., Yaphank, NY, USA

2.5 Acceptor Photobleach FRET

Förster resonance energy transfer (FRET) is the non-radiative transfer of energy from the donor fluorescent molecule, upon excitation, to the acceptor fluorescent molecule. When the two molecules are in close proximity i.e. the distance between the acceptor and the donor is less than or equal to 10nm, excitation of the donor molecule results in a transfer of energy from the donor to the acceptor, which is later emitted at a characteristic emission wavelength of the acceptor[69]. Acceptor photobleach FRET employs a backward approach, wherein selective photochemical destruction of the acceptor fluorophore is used. If the two fluorophores had previously been close enough to FRET, there is an increase in the donor emission[70].

Acceptor photobleach FRET was conducted using a customized Inverted Epifluorescence Microscope (Olympus IX-81 Base scope running Slidebook Version 6.0) with a 60X/0.90 N/A (Olympus, Center Valley, PA, USA) air objective. The camera used for acquisition was Hamamatsu Flash 4.0 v1 CMOS (Hamamatsu Corporation, Bridgewater, NJ, USA). The images were acquired at excitation 387 nm +/- 11 (Semrock FF01-387/11-25, Rochester, NY, USA) and emission 520 nm +/- 35 (Semrock FF01-520/35-25, Rochester, NY, USA) for DAPI; excitation 560 nm +/- 25 (Semrock FF01-560/25-25, Rochester, NY, USA) and emission 607nm +/- 36 (Semrock FF01-607/36-25, Rochester, NY, USA) for 5HT1AR (donor); excitation 650 nm +/- 13 (Semrock FF01-650/13-25, Rochester, NY, USA) and emission 684nm +/- 24 (Semrock FF02-684/24-25, Rochester, NY, USA) for SUMO-1 (acceptor) in conjunction with Semrock penta band dichroic (Semrock, FF408/504/581/667/762-Di01-35*36, Rochester, NY, USA).

Acceptor photobleaching was performed by first acquiring images for DAPI and donor before photobleach, followed by photobleaching in the acceptor channel for 30 seconds, and

acquiring images for both the donor and the acceptor. This procedure was repeated in the same region as a time-lapse capture for 15 timepoints at 30 second intervals.

2.6 Calculation of FRET Efficiency

Analysis for FRET was performed by using the following equation:

$$\mathbf{E = 1/[1+(r/R_0)^6]}^{[71, 72]}$$

Where, R_0 is the characteristic förster distance for the given donor and acceptor fluorophore at which the FRET efficiency is 50%; r is the distance between the donor and acceptor molecules and E is the FRET efficiency which is calculated from the following equation:

$$\mathbf{E = (Dpost - Dpre)/ Dpost}^{[71, 72]}$$

Where, Dpost is the donor intensity after acceptor photobleaching and Dpre is the donor intensity before acceptor photobleaching. The förster distance for the Alexa Fluor 568 and Alexa Fluor 647 is 8.2 nm and for Alexa Fluor 594 and Alexa Fluor 647 is 8.5 nm

2.7 Confocal Imaging

Images were acquired at the Olympus 3I Spinning Disk Confocal Epifluorescence TIRF Inverted Microscope (Olympus IX-71 Base Scope running SlideBook version 5.5) with a 100X/1.4 N/A oil immersion objective (Olympus, Center Valley, PA, USA). The camera used for acquisition was Andor Zyla 4.2 CMOS (Andor Technology Ltd, Belfast UK). Images were acquired using solid state lasers of 405 nm for DAPI, 488 for WGA, 561 for 5HT1AR and 642 nm for SUMO-1. Images were acquired at each Z-step of 25nm. The processing of images and colocalization analysis were performed using CellProfiler (developed by Broad Institute's

Imaging Platform, Cambridge, MA, USA). To analyze the colocalization of 5HT1AR and SUMO-1, both Pearson's and Mander's coefficients were determined using CellProfiler.

2.8 Statistical Analysis

All statistical analyses were conducted using GraphPad Prism software. Shapiro-Wilk's test for normality and Brown-Forsythe-Levene test for homogeneity of variance were used to determine if the data met the requirements for a parametric analysis of variance. Transformation of data also did not result in meeting the criteria for normality and homogeneity of variance. Thus, a non-parametric test i.e. Kruskal-Wallis was conducted followed by Dunn's multiple comparison's test. All data are represented as mean +/- SEM.

3. RESULTS

3.1 Characterization of FRET antibody pair

To identify a pair of 5HT1AR and SUMO-1 antibodies that would FRET, we used N2A cells which endogenously express the 5HT1ARs and the SUMO-1 machinery and have been previously used in our lab to study SUMOylation of 5HT1ARs. We used antibodies that recognize different epitopes on the 5HT1ARs and SUMO-1 to perform acceptor photobleach FRET. The different antibodies used were raised against different peptides in the amino acid sequence of 5HT1AR and SUMO-1, the different peptides are: 5HT1AR (400-422aa), 5HT1AR (294-312aa), SUMO-1 (1-101aa) and SUMO-1 (85-97aa). The 5HT1AR (400-422aa) antibody was observed to colocalize with SUMO-1 (1-101aa) antibody but did not FRET (Figure 1A). The 5HT1AR (294-312aa) antibody was observed to FRET with SUMO-1(1-101aa) antibody in the cell membrane, as indicated by an increase in the donor (5HT1AR) channel intensity with a decrease in the acceptor (SUMO-1) channel intensity (Figure 1B and 1C). The calculated distance between the fluorophores was, 13.6 nm, and was calculated from an average of three regions of interest on the cell membrane per cell from four cells in the field of view. To reduce the distance between the two fluorophores, we tried a different antibody for SUMO-1 which recognized amino acid sequence 85-97 on the SUMO-1 protein. We performed acceptor photobleach FRET with 5HT1AR antibody (294-312 aa) and SUMO-1 antibody (85-97 aa) and the pair of antibodies was observed to FRET in the cell membrane and peri-nuclear regions. The distance between the fluorophores was observed to be in the range of 8.4 – 9.6 nm (Figure 1D

and 1E). All the above experiments were performed using full-length secondary antibodies conjugated to fluorophores.

To further reduce the distance between the fluorophores we tried using Fab' fragment of the secondary antibodies conjugated to fluorophores and performed acceptor photobleach FRET using the same pair of primary antibodies. We observed that using the Fab' fragments increased the distance between the fluorophores, as the distance was calculated as 11.1-12.0 nm (data not shown). This suggested that the two secondary antibodies were either at a distance such that having full-length sequences helped reduce the distance between the two fluorophores or the orientation of the full-length secondary antibodies reduced the distance between the fluorophores as compared to their Fab' fragment counterparts. Hence, our results demonstrated that the best pair of antibodies for observing acceptor photobleach FRET are 5HT1AR (294-312 aa) with SUMO-1 (85-97 aa) and full-length secondary antibodies.

3.2 Identification of primary SUMOylation site on the 5HT1AR

To identify the primary site of SUMOylation on the 5HT1AR, we used HEK293 cells which do not endogenously express the 5HT1ARs but express SUMO-1 machinery although with low levels of Ubc9. We co-transfected HEK293 cells with the wild type 5HT1AR and Ubc9 constructs and performed acceptor photobleach FRET using the antibodies characterized in the previous experiments. We observed that the 5HT1ARs were expressed in the cell membrane and the perinuclear regions, and they did FRET with SUMO-1 in both the regions (Figure 2A and 2B). The distance was calculated to be within the range of 8.3-10.9 nm and was calculated from 4 regions of interest per cell and four cells per field of view. The distances observed in HEK293 cells showed a lot of variability within one experiment and between the three experiments. To avoid this variability, we tried a neuronal cell line, SHSY5Y cells which also do not express

endogenous 5HT1ARs but express the SUMO-1 machinery. We transfected SHSY5Y cells with wild type 5HT1AR construct and performed acceptor photobleach FRET using the same set of antibodies previously characterized. We observed a similar pattern of labelling of the 5HT1ARs as was seen in HEK293 cells, and the FRET distances were calculated to be within the range of 7.8-8.6 nm (Figure 2C and 2D). The variability of the distances was very low as compared to HEK293 cells. Furthermore, the FRET distances were less as compared to what we had observed in the N2A cells, which endogenously express 5HT1ARs.

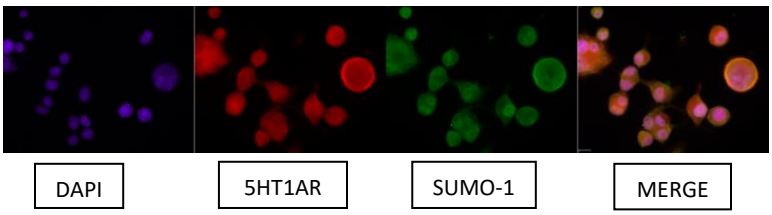
To determine the primary site of SUMOylation on 5HT1ARs, we next transfected SHSY5Y cells with either the wild type 5HT1AR or one of the mutant 5HT1AR constructs and performed acceptor photobleach FRET. Each group was performed in triplicate in each experiment and each experiment was repeated three times. The FRET distances for the K324R, K232.235R and K232.235.324R mutants were greater compared to the wild type 5HT1AR control as demonstrated by Kruskal-Wallis test ($p<0.0001$) followed by Dunn's multiple comparisons test. The data were found to be not normal and transformation of data also did not meet the criteria of normality, thus we performed non-parametric test i.e. Kruskal-Wallis test followed by Dunn's post hoc test. The FRET distances for K332R, K302R and K302.332R mutants were not significantly different compared to the wild type control (Figure 3).

3.3 The effect of acute agonist stimulation of 5HT1ARs on the SUMOylated 5HT1ARs

To study the effect of agonist stimulation of 5HT1ARs on the SUMOylated 5HT1ARs, we used the N2A cells and treated them with 0.2 mg/ml of 8-OH-DPAT for 5 min, 15 min, 30 min, and 60 mins. Immunolabelling was performed using 5HT1AR (400-422aa) and SUMO-1 (85-97 aa) antibodies followed by full length secondary antibodies. Wheat germ agglutinin and DAPI were used as markers for the cell membrane and the nucleus respectively (Figure 4A).

Each treatment condition was performed in duplicate in each experiment and each experiment was repeated three times. The cells were imaged using confocal microscopy and colocalization analysis was performed using CellProfiler. Interestingly, both the Pearson's and Mander's coefficients were observed to be 0.97 and 1.0 respectively for all the groups. In our previous experiments, these antibodies were found to not FRET, so it was clear that the distance between the two fluorophores was more than 20 nm. The lowest possible distance we could resolve between two objects using the 100X/1.40 N/A oil immersion objective was around 100 nm. This led us to hypothesize that the two fluorophores, although not close enough to FRET, were too close to be resolved by the objective, thus explaining the high correlation observed with the Pearson's and Mander's coefficients.

A.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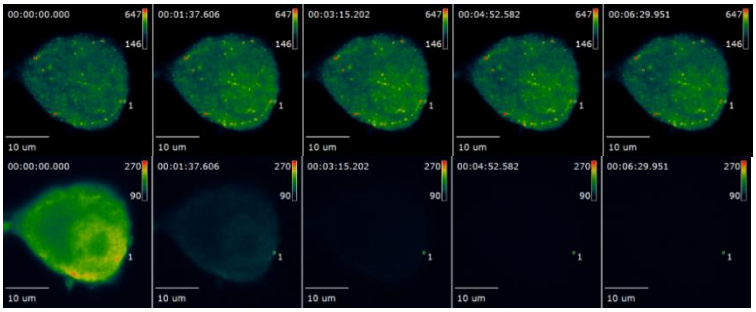
SUMO-1
MERGE

B.



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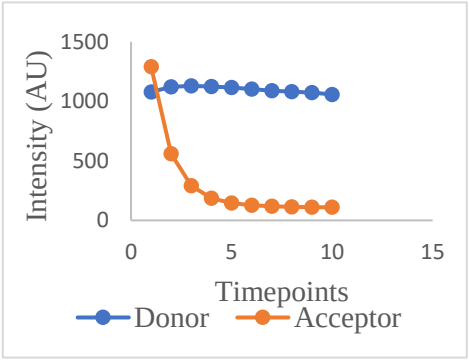
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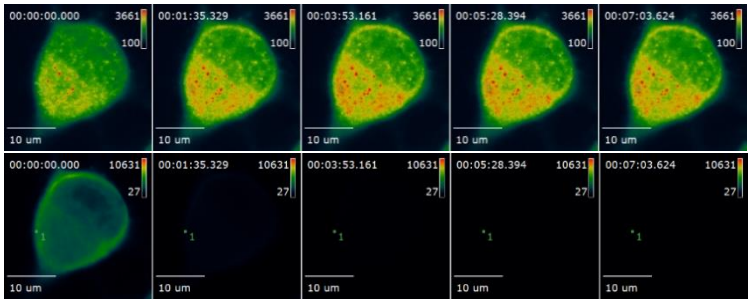
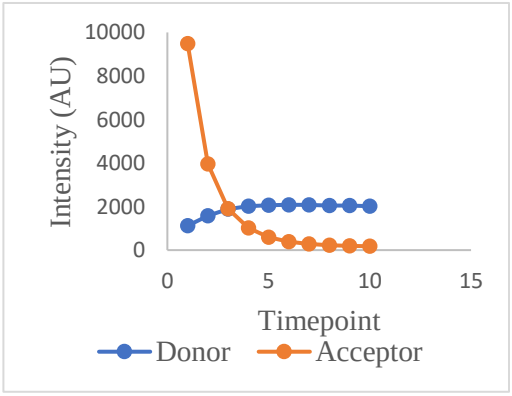
Acceptor

D.



E.


10000
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6000
4000
2000
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Intensity (AU)
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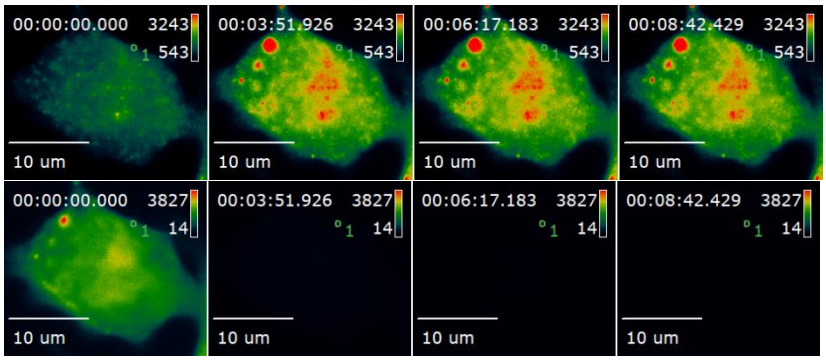
15
Timepoint
Donor
Acceptor

Figure 1: Characterization of FRET antibody pair (A) Immunolabelling of N2A cells with 5HT1AR antibody (400-422aa) and SUMO-1 (1-101aa) showing colocalization of 5HT1AR with SUMO-1. This pair of antibodies were not found to FRET. The scale bar is 10 µm and is indicated in the image with the merged channels. **(B)** Immunolabelling of N2A cells with 5HT1AR antibody (294-312aa) and SUMO-1 (1-101aa); this pair of antibodies were found to FRET with each other and the distance between the fluorophores was calculated as 13.6 nm. Donor channel (5HT1AR antibody with Alexa Fluor 568) in pseudocolour in the top panel and acceptor channel (SUMO-1 antibody with Alexa Fluor 647) in the lower panel are shown before acceptor photobleaching (left) and at 3^{rd}, 5^{th}, 7^{th} and 9^{th} photobleach (left to right). **(C)** Representative graph of the FRET efficiency for the given donor-acceptor pair indicating the increase in donor channel intensity with a decrease in acceptor channel intensity. The FRET efficiency was calculated to be 4.5%. **(D)** Immunolabelling of N2A cells with 5HT1AR antibody (294-312aa) and SUMO-1 (85-97aa); this pair of antibodies were also found to FRET and the distance between the fluorophores was lower as compared to last pair and was calculated as 8.41 nm. Donor channel (5HT1AR antibody with Alexa Fluor 568) in pseudocolour in the top panel and acceptor channel (SUMO-1 antibody with Alexa Fluor 647) in the lower panel are shown before acceptor photobleaching (left) and at 3^{rd}, 5^{th}, 7^{th} and 9^{th} photobleach (left to right). **(E)** Representative graph of the FRET efficiency for the given donor-acceptor pair indicating the increase in donor channel intensity with a decrease in acceptor channel intensity. The FRET efficiency was calculated to be 46.2%. All the images were acquired using 60X/0.90 N/A air objective. The scale bar is 10µm.

A.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00:08:42.429 3243 543
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10 um

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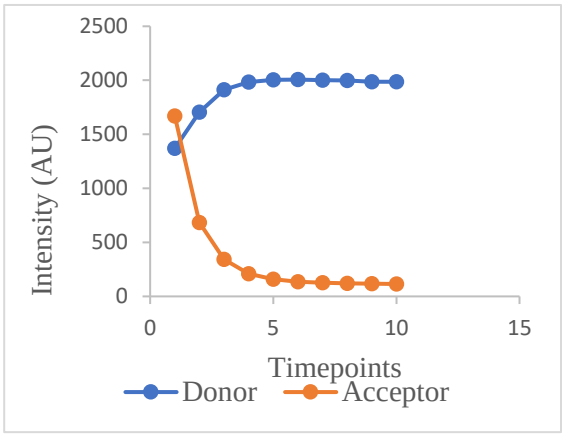
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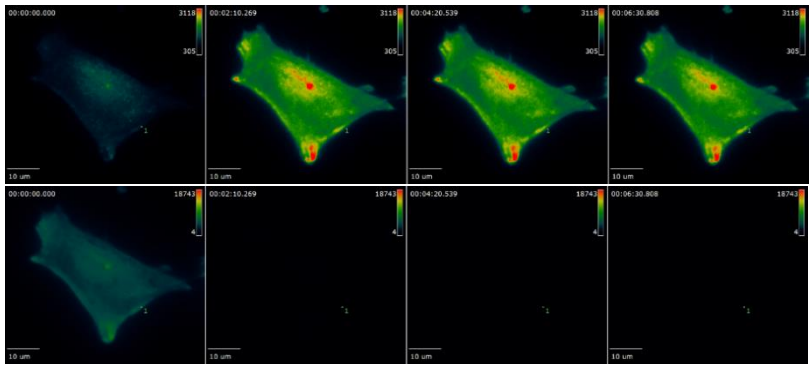
Intensity (AU)
0
5
10
15
Timepoints
Donor
Acceptor

C.



D.

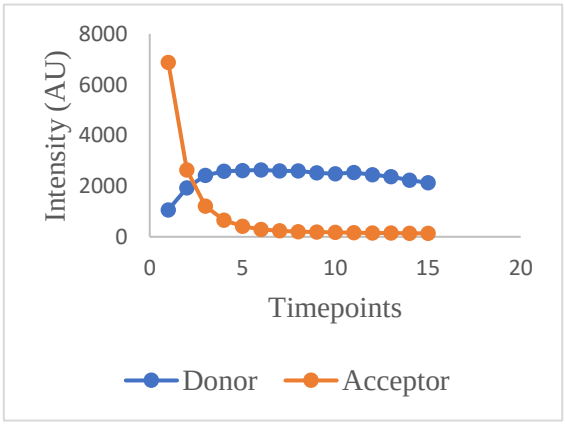

8000
6000
4000
2000
0
Intensity (AU)
0
5
10
15
20
Timepoints
Donor
Acceptor

Figure 2: Acceptor photobleach FRET in HEK293 and SHSY5Y cells transfected with wild type 5HT1ARs. (A) Immunolabelling of HEK293 cells with 5HT1AR antibody (294-312aa) and SUMO-1 (85-97aa); this pair of antibodies were found to FRET and the distance between the fluorophores was calculated as 8.48 nm. Donor channel (5HT1AR antibody with Alexa Fluor 568) in pseudocolour in the top panel and acceptor channel (SUMO-1 antibody with Alexa Fluor 647) in the lower panel are shown before acceptor photobleaching (left) and at 4^{th}, 7^{th} and 10^{th} photobleach (left to right). **(B)** Representative graph of the FRET efficiency for the given donor-acceptor pair indicating the increase in donor channel intensity with a decrease in acceptor channel intensity. The FRET efficiency was calculated to be 44.9%. **(C)** Immunolabelling of SHSY5Y cells with 5HT1AR antibody (294-312aa) and SUMO-1 (85-97aa); this pair of antibodies was found to FRET and the distance between the fluorophores was calculated as 7.68 nm. Donor channel (5HT1AR antibody with Alexa Fluor 568) in pseudocolour in the top panel and acceptor channel (SUMO-1 antibody with Alexa Fluor 647) in the lower panel are shown before acceptor photobleaching (left) and at 5^{th}, 9^{th} and 13^{th} photobleach (left to right). **(D)** Representative graph of the FRET efficiency for the given donor-acceptor pair indicating the increase in donor channel intensity with a decrease in acceptor channel intensity. The FRET efficiency was calculated to be 59.7%. All the images were acquired using 60X/0.90 N/A air objective. The scale bar is 10µm.

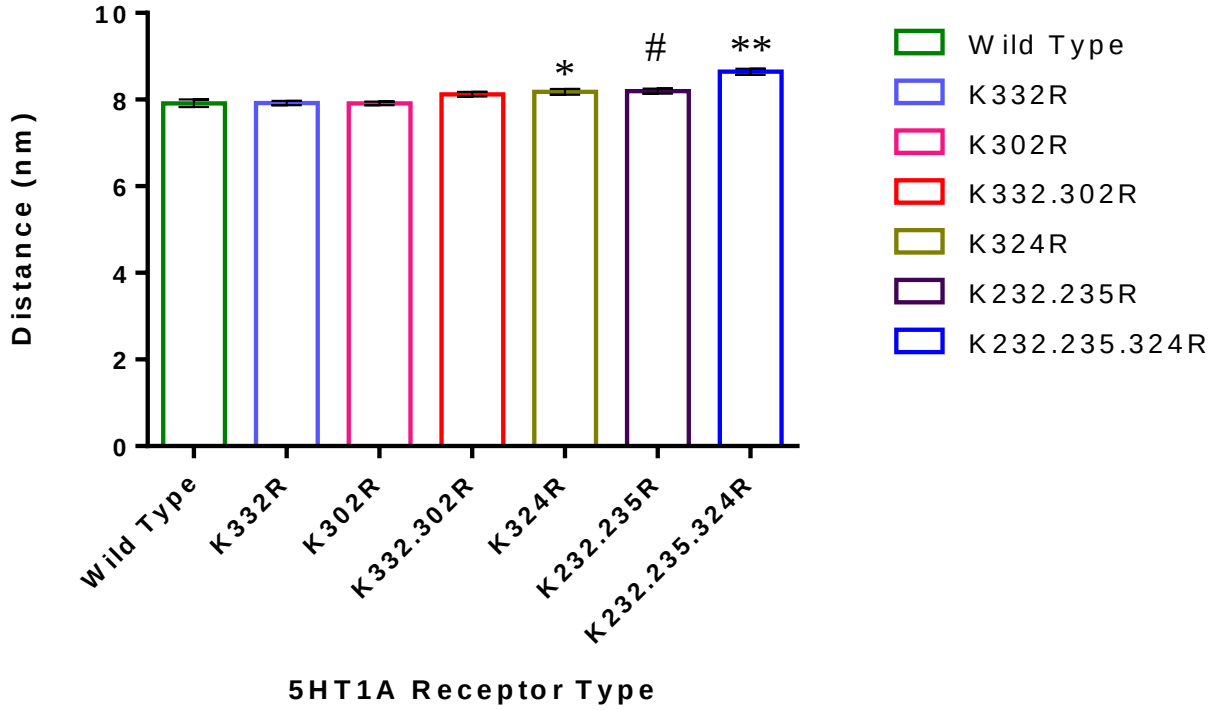
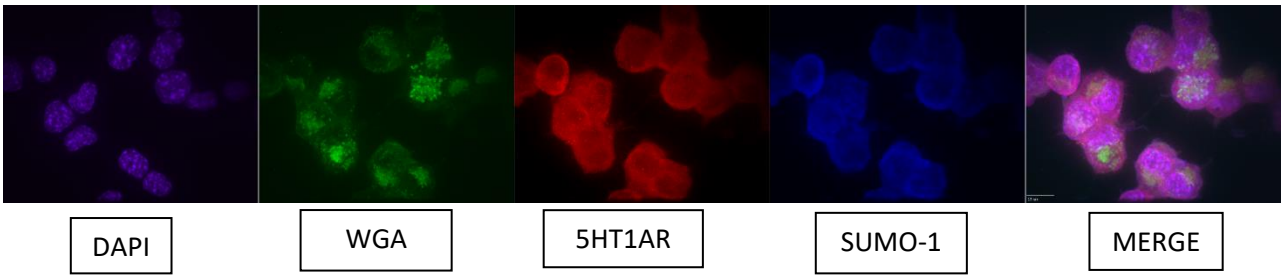


Figure 3: Acceptor photobleach FRET in SHSY5Y cells transfected with wild type 5HT1ARs vs mutant 5HT1ARs. The above graph depicts the distances between the fluorophores attached to 5HT1AR antibody and SUMO-1 antibody in wild type 5HT1ARs vs the mutant 5HT1ARs. The data did not pass the test for normality and transformation also did not normalize the data; thus Kruskal-Wallis non-parametric test was performed. p value for Kruskal-Wallis non-parametric test was observed to be <0.0001. Dunn's multiple comparisons test was conducted following Kruskal-Wallis test and the adjusted p value was observed to be 0.0065 (*) for Wild Type Vs K324R, 0.0057(#) for Wild Type Vs K232.235R and <0.0001(**) for Wild Type Vs K232.235.324R.

A. VEHICLE-TREATED GROUP



B. AFTER 60 MINS TREATMENT WITH 8-OH-DPAT

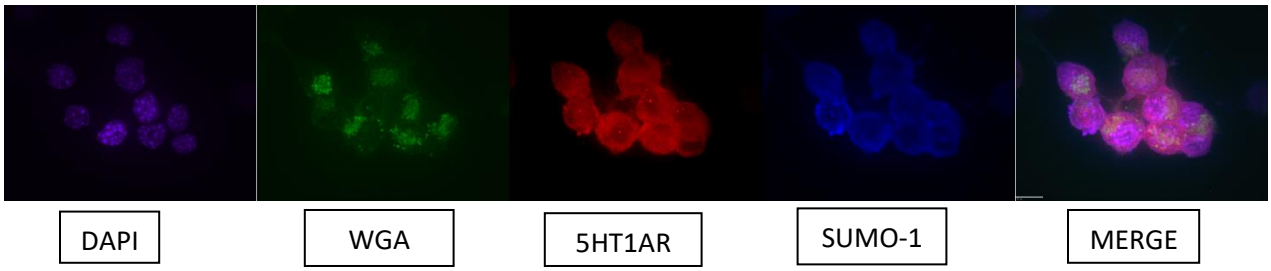


Figure 4: Effect of agonist stimulation of 5HT1ARs on the SUMOylation of 5HT1ARs.

(A) Immunolabelling of N2A cells using 5HT1AR antibody (400-422aa) and SUMO-1 (85-97aa) antibody along with wheat germ agglutinin (WGA) and DAPI for staining membranes and nucleus respectively, in cells which were treated with vehicle and **(B)** cells after 60 minutes of treatment with 0.2 mg/ml 8-OH-DPAT. Mander's coefficients for the vehicle-treated were found to be 0.95 (k1: SUMO-1 to 5HT1AR) and 1 (k2: 5HT1AR to SUMO-1). Mander's coefficients for the 60 mins treatment group were also found to be 0.95 (k1: SUMO-1 to 5HT1AR) and 1 (k2: 5HT1AR to SUMO-1). Therefore, both the treated and vehicle-treated groups had similar extent of colocalization. All the images were acquired using 100X/1.40 N/A oil immersion objective. The scale bar is 10 µm.

4. DISCUSSION

In this study, we for the first time report the SUMOylation of 5HT1ARs in the plasma membrane using an acceptor photobleach FRET based approach. We used cell lines expressing both endogenous 5HT1ARs (N2A) and transfected 5HT1ARs (HEK293 and SHSY5Y) and observed SUMOylation of 5HT1ARs in the cell membrane in all the cell lines. Previous studies in our lab had reported that 5HT1ARs are SUMOylated in the membrane fractions of homogenates from various brain regions in rat such as cortex, hypothalamus and dorsal raphe[73]. SUMOylation of 5HT1ARs was also previously observed in the membrane fractions from N2A cells, a mouse neuroblastoma cell line that express endogenous 5HT1ARs[74]. The previous studies suggested SUMOylation of 5HT1ARs in the plasma membrane using cell fractionation and immunoprecipitation techniques. As opposed to the molecular biology-based techniques used previously to study the SUMOylation of 5HT1ARs, we used an acceptor photobleach FRET-based approach to confirm the interaction between the 5HT1ARs and SUMO-1 at the plasma membrane.

In this study, we observed a robust labelling and colocalization of 5HT1ARs and SUMO-1 in regions around the nucleus, suggesting the presence of SUMOylated 5HT1ARs in the ER and TGN, since ER and TGN are usually present around the nucleus. The 5HT1AR (294-312aa) and SUMO-1 (85-97aa) antibodies were also found to FRET in these regions. Previous studies in our lab had shown that SUMOylated 5HT1ARs are colocalized with markers for ER and TGN in subcellular fractionations of rat cortex homogenates[73]. TGN and ER are known to be involved in the transportation of many cellular proteins to different compartments of the cell. Many transmembrane proteins such as receptors, transporters and proteases are known to undergo internalization upon stimulation by various ligands. After internalization proteins are

transported to early endosomes. From early endosomes, transport continues to late endosomes and eventually the proteins undergo one of the following fates: (1) transported to lysosomes for degradation, (2) recycled back to the plasma membrane, or (3) transported to the TGN[75, 76]. Transport to the plasma membrane is divided into the fast, direct pathway and the slower pathway, going via recycling endosomes. Transport to the TGN may go through recycling endosomes or even late endosomes, and some proteins continue towards the ER[75, 77-79]. Other than the aforementioned pathway, newly synthesized proteins are known to enter the biosynthetic-secretory pathway in the ER by crossing the ER membrane from the cytosol. During their subsequent transport, these proteins move from the ER to the golgi apparatus and then to the TGN to get sorted to the cell surface and elsewhere. These newly synthesized proteins pass through a series of compartments in the golgi complex where they undergo post-translational modifications[80, 81]. The presence of SUMOylated 5HT1ARs in the TGN and ER suggests that either SUMOylation leads to the trafficking of 5HT1ARs from the plasma membrane to the TGN and ER or SUMOylation is a post-translational modification that is occurring in the TGN and ER, which results in the sorting of the modified 5HT1AR to the plasma membrane. Therefore, our data provides further evidence for the involvement of SUMOylation in the trafficking of 5HT1AR either to the TGN and ER from the cell membrane or vice-versa.

We used antibodies recognizing different epitopes on the 5HT1ARs and SUMO-1 to study the interaction and distance between the two proteins. 5HT1AR is a 422aa transmembrane protein, with three extracellular and three intracellular loops[82]. The third intracellular loop of the 5HT1AR (219-343 aa) is important for the coupling of G-proteins and contains sites for post-translational modifications of the 5HT1AR such as phosphorylation[83]. Our lab previously used SUMO-prediction programs SUMOplot (Abgent, San Diego, CA, USA) and GPS-SUMO[84] to

predict putative sites of SUMOylation on the 5HT1AR. The predicted sites were found to lie in the third intracellular loop of the 5HT1AR and one of the predicted lysine residue at 302aa was found to lie in the antigen recognition sequence of the 5HT1AR (294-312aa) antibody. Our data from the endogenous 5HT1ARs in N2A cells also suggests that the primary site of SUMOylation is very close to the antigen recognition sequence of 5HT1AR antibody (294-312aa) since the distance between 5HT1AR antibody and SUMO-1 antibody was in the range of 8.4 – 9.6 nm. Thus, this data indicates that the primary site of SUMOylation on 5HT1AR is very close to the 294-312 aa sequence and lies in the third intracellular loop of the 5HT1AR.

Furthermore, we observed that using the full-length secondary antibodies resulted in lower distances between the fluorophores as compared to the Fab' fragments of the secondary antibodies. This was interesting, because generally using a Fab' fragment decreases the distance between the fluorophores compared to the full-length secondary, but our data indicates the opposite, where we observe an increase in the distance between fluorophores with Fab' fragments[85]. This could be due to one of the following reasons, first, the primary antibodies for 5HT1AR and SUMO-1 are further away from each other such that the full-length sequence of the secondaries helps reduce the distance between the fluorophores. Second, the primary antibodies are close enough but are bound at an orientation such that the full-length secondary antibodies help bring the fluorophores closer as compared to their Fab' fragment counterparts. Third, the Fab' fragments are binding in such an orientation that the distance between the fluorophores increases as compared to the full-length secondary antibodies. Therefore, our results suggest that one of the above factors could be contributing to the increased distance we observe with the Fab' fragments as compared to the full-length secondary antibodies.

Next, we used HEK293 cells, which do not express 5HT1AR but have been shown to have all the components of SUMO machinery but with low levels of Ubc9[86-88]. HEK293 are human embryonic kidney cells which have been used by many researchers to study the signal transduction of many neuronal proteins[89-92]. We observed that the transfected 5HT1ARs also FRET with SUMO-1 in HEK293, although there is a lot of variation in the FRET distances within each experiment. This variation in distances can be due to the difference in the membrane composition of the neuronal cells and embryonic kidney cells. Membrane cholesterol is known to stabilize the expression of 5HT1ARs and facilitate ligand binding and signal transduction of 5HT1ARs[93-96]. One possibility could be the difference in the membrane cholesterol in HEK293, results in a non-homogeneous conformation of 5HT1ARs on the cell membrane. To reduce this variability in the FRET distance, we tried a neuronal cell line which does not express endogenous 5HT1ARs but contains the SUMO machinery, i.e., SHSY5Y cells[97-100]. The transfected 5HT1ARs in SHSY5Y were found to FRET with SUMO-1, and the variation in distances within each experiment and between different experiments was reduced.

SHSY5Y cells were transfected with either the wild type 5HT1AR or one of the mutant 5HT1ARs to observe any change in the FRET between the 5HT1AR and SUMO-1 upon mutation of lysines residues in predicted SUMO sites. We observed no significant difference in the distances for K332R, K302R and K302.332R mutants compared to the wild type 5HT1AR. This indicates that the lysine residues at 302 and 332 aa are not SUMOylated. However, we observed a significant increase in distance with K324R, K232.235R and K232.235.324R mutants. This suggests that lysine residues at 232, 235 and 324 aa are involved in SUMOylation. SUMOylation is known to occur at multiple sites on a substrate and if the primary site of SUMOylation is mutated, other nearby lysine residues act as secondary sites and get

SUMOylated[51, 101]. This could be the phenomenon that we are observing with the mutations at 232, 235 and 324 lysine residues, where one of these sites could be the primary site of SUMOylation and due to its mutation other nearby lysine residues might be undergoing SUMOylation. If the primary site of SUMOylation on 5HT1AR had been mutated, we would have expected to observe no FRET or a very large increase in the distance between the mutant 5HT1AR and SUMO-1. But since we observe a significant but small increase in the distance between the mutant 5HT1AR and SUMO-1, another possibility could be that the lysines at 232, 235 and 324 although are involved in the SUMOylation of 5HT1AR, are not the primary sites of SUMOylation. The increase in distance observed with K232.235R, K232.235.324R and K324R can also be due to a change in the conformation of the 5HT1AR after the mutations, which resulted in an increase in the distance between the binding site of SUMO-1 and 5HT1AR antibody. Moreover, we did not test the single mutants of the lysines at 232 and 235, which would have helped in elucidating the effect of these single mutations on the interaction between 5HT1AR and SUMO-1. To compliment the acceptor photobleach FRET approach, we could use immunoprecipitation techniques to observe the difference in the levels of SUMOylated receptors in wild type controls vs the lysates with mutant receptors. Further studies are needed to resolve the primary sites of SUMOylation.

In this study, we conducted an immunocytochemistry-based approach to observe the effect of agonist stimulation of 5HT1ARs on the SUMOylation of 5HT1ARs in the cell membrane. We observed a very high correlation between the 5HT the membrane fractions of rat cortex homogenates[73]. To compliment the above findings, we used an immunocytochemistry-based approach and specifically looked at the effect of agonist stimulation of 5HT1ARs in the cell membrane. To study the effect of agonist stimulation of 5HT1ARs on the SUMOylation of

5HT1ARs, we treated N2A cells with 0.2 mg/ml 8-OH-DPAT for different timepoints and conducted immunocytochemistry experiments. The dose was selected based on the previous experiments conducted using rat cortex[73]. The time points for treatment were chosen based on the pharmacodynamics of 8-OH-DPAT and the treatment times used to observe effect of agonist stimulation in earlier studies[102, 103].. Many researchers have studied the effect of acute and chronic agonist treatment on 5HT1AR desensitization in cell culture also. Chronic treatment of Swiss 3T3 cells transfected with 5HT1ARs for 24 hours with 5-HT and of CHO-K1 cells stably expressing 5HT1ARs for 24 hours with 8-OH-DPAT was observed to induce receptor desensitization[104, 105]. Whereas acute treatment for 10 minutes with 8-OH-DPAT of transfected Hela cells lead to rapid uncoupling of the receptor from G proteins and from the inhibition of adenylyl cyclase[106]. The effects of acute agonist stimulation of 5HT1AR appear to depend on PKC and the phosphorylation of 5HT1AR[106]. Similar to the above studies, we wanted to determine the effect of acute agonist stimulation of 5HT1AR on its SUMOylation, thus we performed a time course experiment keeping in mind the pharmacodynamics of 8-OH-DPAT and timepoints at which earlier studies had observed changes in receptor function. We treated our cells for 5 mins, 15 mins, 30 mins and 60 mins with 0.2 mg/ml of 8-OH-DPAT. Immunolabelling was performed using 5HT1AR (400-422aa) and SUMO-1 (85-97aa). This pair of antibodies was found to colocalize but not FRET in the previous experiments. The correlation coefficients for each treatment group and the group were 0.95 and 1.0 respectively. This indicated a very high correlation between the 5HT1AR and SUMO-1 antibody, which was very unusual as it is very difficult to observe this high of a correlation between two interacting molecules[107]. Since this pair of antibodies had previously been found to not FRET, the distance between fluorophores was greater than 20 nm, but the next question was whether the distance

was larger than 100 nm. Using a 100X/1.40 N/A oil immersion objective, the lowest resolvable distance between the two objects using a light microscope is around 100nm. Therefore, any pair of objects that lie closer than 100 nm would be detected as one, and the correlation coefficients for such objects would be 1. This would explain the high correlation that we observed with the aforementioned pair of antibodies. To test this hypothesis, we conducted an electron microscopy experiment to confirm whether the distance between two fluorophores is indeed less than 100 nm.

In conclusion, this study reported that the 5HT1ARs are SUMOylated in the plasma membrane using an acceptor photobleach FRET-based approach. We found three probable lysine residues on the 5HT1AR (232, 235 and 324) that are possibly involved in SUMOylation. However, further studies will be needed to identify whether one of them was a primary site of SUMOylation or if there is another lysine residue that is the primary SUMOylation site on the 5HT1ARs. Identification of the primary site of SUMOylation would provide us with a tool to study how SUMOylation regulates 5HT1AR function and its role in receptor desensitization. We also observed robust labelling of the SUMOylated 5HT1ARs in the regions near the nucleus and the 5HT1AR (294-312aa) and SUMO-1 (85-97aa) antibodies seem to FRET in those regions. Further studies would be needed to label these regions using a TGN or ER marker and perform acceptor photobleach FRET between 5HT1AR and SUMO-1 in these regions to confirm the presence of SUMOylated 5HT1ARs in the TGN and ER. This along with the identification of the primary site of SUMOylation on 5HT1AR would help us understand the stage at which 5HT1ARs get SUMOylated. This knowledge would help us elucidate whether the 5HT1ARs are SUMOylated in the TGN and then transported to the plasma membrane or are they SUMOylated at the plasma membrane and then trafficked to the TGN and ER. Furthermore, studies need to be

performed to observe the effect of agonist stimulation on the SUMOylation of 5HT1ARs using high resolution microscopy to avoid the limitations of light microscopy. These studies would help us understand the agonist-induced desensitization of 5HT1ARs and the role of SUMOylation in this process. Together, all the above studies would help us elucidate the role SUMOylation plays in the regulation of 5HT1AR function and whether 5HT1AR SUMOylation is involved in agonist induced 5HT1AR desensitization.

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